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Classification of aster yellows-group phytoplasmas based on combined analyses of rRNA and *tuf* gene sequences

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Seventy phytoplasma isolates, including 10 previously characterized reference strains, of the aster yellows group were examined by RFLP analysis of PCR-amplified rDNA and RFLP and sequence analysis of the *tuf* gene. On the basis of rDNA restriction profiles, seven previously proposed 16S rDNA subgroups (16SrI-A, -B, -C, -D, -E, -F and -K) were recognized in the material examined. In addition, three new subgroups that differ in the RFLP profiles were identified and designated 16SrI-L, 16SrI-M and 16SrI-N. Of the two types of rDNA sequences used, an 1800 bp fragment comprising the entire 16S rRNA gene and the 16S–23S rDNA spacer region proved more suitable for AY-group phytoplasma differentiation than a 1240 bp fragment of the 16S rRNA gene. Many differences in the rDNA profiles between the subgroups could be explained by sequence heterogeneity of the two phytoplasmal rRNA operons. The subgroups delineated by RFLP analysis of a 940 bp *tuf* gene fragment are consistent with subgroups defined on the basis of rDNA sequences. However, subgroups 16SrI-D, -L and -M showed the same *tuf* gene restriction profiles as subgroup 16SrI-B. This result was confirmed by sequence analysis in which these subgroups differed slightly in their *tuf* gene sequence, when compared with members of subgroup 16SrI-B. On the basis of combined analyses of rDNA and *tuf* gene sequences and in view of pathological aspects, the taxonomic distinction of AY-subgroups 16SrI-A, -B, -C, -D, -E, -F, -K and -N appears to be substantial.

Keywords: phytoplasmas, aster yellows, *tuf* gene, rDNA, phylogeny

INTRODUCTION

Traditionally, diseases caused by the unculturable phytoplasmas were named, and the identity of the associated agents determined, mainly on the basis of the plant hosts infected and the symptoms induced. In recent years, both serological and DNA-based techniques have been employed to identify and differentiate phytoplasmas. Comprehensive classification of phytoplasmas was first accomplished by RFLP analysis of the conserved 16S rRNA gene (Lee *et al*., 1993b; Schneider *et al*., 1993). The phytoplasma groups delineated using RFLP analysis largely matched phytoplasma subclades delineated by phylogenetic analysis of full-length or nearly full-length sequences of 16S rDNA. Thus far, 16S rDNA from more than 60 phytoplasma strains has been sequenced and analysed (Namba *et al*., 1993; Gundersen *et al*., 1994; Seemüller *et al*., 1994, 1998). Phylogenetic analyses showed that the phytoplasma clade can be divided into at least 20 subclades, of which several include more than one putative taxonomic unit (Seemüller *et al*., 1998). The subclades were proposed to be delineated at species level, using the provisional taxonomic prefix *Candidatus* (Bradbury, 1997).

The aster yellows (AY) group is the largest of the phytoplasmas: more than 100 isolates have been

Abbreviation: AY, aster yellows; see Table 1 for phytoplasma strain abbreviations.

The EMBL/GenBank accession numbers for the *tuf* gene sequences of the aster yellows phytoplasmas examined are AJ271306 (strain AV2192), AJ271307 (AVUT), AJ271308 (AYA), AJ271309 (BB), AJ271310 (BBS3), AJ271311 (CVB), AJ271312 (DEV), AJ271313 (HYDP), AJ271314 (IPWB), AJ271315 (IOWB), AJ271316 (IRaP), AJ271317 (KVF), AJ271318 (KVM), AJ271319 (PaWB), AJ271320 (PRIVA), AJ271321 (PVM), AJ271322 (PVW), AJ271323 (SAY) and AJ271324 (STRAWB2).

Table 1. Classification of AY phytoplasmas based on RFLP analysis of PCR-amplified rDNA and *tuf* gene sequences

Previous or alternative strain acronyms are given in parentheses.

Phytoplasma isolate*	Disease caused and/or natural host	Country/state	RFLP classification†		Reference (phytoplasma origin)
			16SrI subgroup	*tuf* gene subgroup	
BB*	Tomato big bud	AR, USA	A	A	Lee *et al.* (1993b)
AY27*	Alberta aster yellows	Canada	A	A	Lee *et al.* (1993b)
PVM	Virescence of *Plantago coronopus*	Germany	A	A	Schneider *et al.* (1993)
CHRY	Yellows of *Chrysanthemum frutescens*	Germany	A	A‡	Obtained from R. Marwitz
CHRYM	Yellows of *Chrysanthemum frutescens*	Germany	A	A‡	Obtained from R. Marwitz
COL	Latent in *Cuscuta odorata*	Germany	A	A‡	Schneider *et al.* (1993)
HYDP	Hydrangea phyllody	Belgium	A	A‡	Schneider *et al.* (1993)
PRIVB	Virescence of primrose (*Primula* sp.)	Germany	A	A‡	Schneider *et al.* (1993)
SAY*	Severe Western aster yellows (celery)	CA, USA	B	B	Lee *et al.* (1993b)
MBS*	Maize bushy stunt	OH, USA	B	B	Gundersen *et al.* (1996)
HyPH1*	Hydrangea phyllody	Italy	B	B	Lee *et al.* (1993b)
HYDB	Hydrangea phyllody	Belgium	B	B	Schneider *et al.* (1993)
HYDF	Hydrangea phyllody	France	B	B	Cousin *et al.* (1986)
AAY	American aster yellows	FL, USA	B	B	Schneider *et al.* (1993)
AWB	American witches'-broom	USA	B	B	Obtained from M. F. Clark
AYM	Aster yellows	France	B	B	Obtained from G. Morvan
AYW	Eastern American aster yellows	NJ, USA	B	B	Schneider *et al.* (1993)
CACT	Cactus aster yellows	USA?	B	B	Obtained from M. F. Clark
CVL	Catharanthus virescence	Peru	B	B	Schneider *et al.* (1993)
CVT	Catharanthus virescence	Thailand	B	B	Schneider *et al.* (1993)
DEV	Virescence of *Delphinium* hybrid	Germany	B	B	Schneider *et al.* (1993)
DIV	Virescence of *Diplotaxis erucoides*	Spain	B	B	Schneider *et al.* (1993)
GLAWB	Gladiolus witches'-broom	Belgium	B	B	Van Slogteren *et al.* (1974)
GLAWC	Gladiolus witches'-broom	France	B	B	Albouy (1966)
PER	Peach with yellowing symptoms	Italy	B	B	Schneider *et al.* (1993)
PRIVC	Virescence of primrose (*Primula* sp.)	Germany	B	B	Schneider *et al.* (1993)
PVW	Virescence of *Plantago major*	Germany	B	B	Schneider *et al.* (1993)
RV	Rape virescence	France	B	B	Schneider *et al.* (1993)
SAFP	Safflower phyllody	Israel	B	B	Schneider *et al.* (1993)
SAS	Sandal spike	India	B	B	Schneider *et al.* (1993)
IPWB**	Italian periwinkle witches'-broom	Italy	B	B	Marcone *et al.* (1997 b)
IPV**	Virescence and phyllody of periwinkle	Italy	B	B	This paper
ILY**	Yellows of lettuce	Italy	B	B	Marcone *et al.* (1997b)
ILY-1**	Yellows of lettuce	Italy	B	B	This paper
LHV**	Virescence and yellowing of hydrangea	Italy	B	B	Marcone *et al.* (1997b)
IHP**	Hydrangea phyllody	Italy	B	B	This paper
ICY**	Yellows of cabbage	Italy	B	B	Marcone *et al.* (1997b)
IBP**	Phyllody of broccoli	Italy	B	B	Marcone *et al.* (1997b)
SIKP**	Phyllody of kale	Italy	B	B	Marcone *et al.* (1997b)
TPV**	Virescence of turnip	Italy	B	B	Marcone *et al.* (1997b)
SICV**	Virescence of *Calendula*	Italy	B	B	Marcone *et al.* (1997a)
PV**	Virescence of field poppy	Italy	B	B	Marcone *et al.* (1997c)
WRY**	Yellows of wild radish	Italy	B	B	Marcone *et al.* (1997c)
IprY**	Yellows of primrose	Italy	B	B	This paper
POY**	Yellows of purslane	Italy	B	B	Marcone *et al.* (1997c)
IraP**	Ranunculus phyllody	Italy	B	B	This paper
ToAY**	Tomato aster yellows	Italy	B	B	This paper
IOY**	Onion yellows	Italy	B	B	This paper
GCV**	Virescence of cyclamen	Germany	B	B	Schneider *et al.* (1997b)

Table 1 *(cont.)*

Previous or alternative strain acronyms are given in parentheses.

Phytoplasma isolate*	Disease caused and/or natural host	Country/state	RFLP classification†		Reference (phytoplasma origin)
			16SrI subgroup	*tuf* gene subgroup	
PoWB**	Witches'-broom of *Populus nigra*	France	B	B	Berges *et al.* (1997)
KVG* (KV)	Clover phyllody	Germany	C	C	Schneider *et al.* (1997b)
KVE	Clover phyllody	UK	C	C	Obtained from M. F. Clark
KVF	Clover phyllody	France	C	C	Faivre-Amiot *et al.* (1970)
KVM	Clover phyllody	France	C	C	Obtained from G. Morvan
GY	Yellows-affected grapevine	Germany	C	C	Maixner *et al.* (1994)
CVA	Leafhopper-borne	Germany	C	C	Schneider *et al.* (1993)
PPT	Potato purple top	France	C	C	Obtained from M. T. Cousin
PaWB*	Paulownia witches'-broom	Taiwan	D	B	Lee *et al.* (1993b)
PaWB-C**	Paulownia witches'-broom	China	D	B	This paper
BBS3*	Blueberry stunt	AR, USA	E	D	Lee *et al.* (1993b)
AYA (ACLR)*	Apricot	Spain	F	E	Schneider *et al.* (1993)
CVB	Leafhopper-borne	Germany	F	E	Schneider *et al.* (1993)
STRAWB2*, **	Strawberry decline	USA	K	F	Jomantiene *et al.* (1998)
AV2192	Aster yellows	Germany	L	B	Schneider *et al.* (1993)
AV2226	Aster yellows	Germany	L	B	Schneider *et al.* (1993)
AV976	Aster yellows	Germany	L	B	Obtained from R. Marwitz
AYBG (BGWL)	Bermudagrass white leaf symptoms	Thailand	L	B	Schneider *et al.* (1993)
PRIVA	Primula yellows	Germany	L	B	Schneider *et al.* (1993)
AVUT	Aster yellows	Germany	M	B	Obtained from R. Marwitz
IOWB	Ipomoea obscura witches'-broom	Taiwan	N	G	Lee *et al.* (1993b)

*/** Reference strains are marked with one asterisk and isolates from original hosts are marked with two asterisks (other isolates were maintained in periwinkle).

† 16SrI subgroup designation according to Lee *et al.* (1993b, 1998); the *tuf* gene subgroup differentiation was based on profiles obtained by digestion with *Sau*3AI, *Alu*I and *Hpa*II (see Table 3).

‡ The *Alu*I profile differs slightly from that of other strains of subgroup A.

examined by several researchers. On the basis of 16S rDNA sequence analysis, the AY group is relatively homogeneous, differing in not more than 2.6% of the nucleotide positions. Despite this low variation, the AY group could be subdivided into several distinct RFLP subgroups (16SrI subgroups) by extensive RFLP analysis of 16S rDNA, employing 15 restriction enzymes (Lee *et al.*, 1993b, 1998; Jomantiene *et al.*, 1998). Further differentiation has been obtained by RFLP analysis of a fragment of a ribosomal protein operon. The combined analysis of both types of ribosomal sequences has led to the most detailed RFLP differentiation, i.e. to the rr-rp subgroups (Gundersen *et al.*, 1996; Jomantiene *et al.*, 1998; Lee *et al.*, 1998). Detailed differentiation of AY phytoplasmas has also been obtained by Southern blot analysis (Lee *et al.*, 1992; Schneider & Seemüller, 1994b) and serological comparisons (Keane *et al.*, 1996).

As an additional tool for the classification of phytoplasmas, the conserved gene encoding the elongation factor TU (*tuf* gene), which has been widely used as a phylogenetic marker (Sela *et al.*, 1989; Cammarano *et al.*, 1992; Ludwig *et al.*, 1993; Ceccarelli *et al.*, 1995; Kamla *et al.*, 1996), was examined by Schneider *et al.* (1997a). By RFLP analysis of the PCR-amplified *tuf* gene using *Sau*3AI restriction endonuclease, 21 AY isolates could be divided into five subgroups. However, the 16S rDNA subgroup affiliation of these isolates according to Lee *et al.* (1993b, 1998) was unknown and the relationship between the *tuf* gene RFLP subgroups and the 16SrI subgroups remained unclear. Thus, the aim of the present work was to determine whether RFLP classification based on the *tuf* gene and that of rDNA would be mutually consistent. As the *tuf* gene shows higher sequence variability than the 16S rRNA gene (Schneider *et al.*, 1997a) and appeared, for this reason, more suitable for differentiation and classification of closely related phytoplasmas, sequence analysis of representative strains of all *tuf* gene RFLP groups was included in this work. Most of the AY isolates we examined were not previously classified, or were insufficiently classified, by either system.

METHODS

Phytoplasma sources. Forty-eight AY phytoplasmas maintained in *Catharanthus roseus* (periwinkle) and 22 isolates collected in the field from various diseased plant species were examined (Table 1). Among the phytoplasmas examined were 10 reference strains which represented all of the 16SrI subgroups (except 16SrI-I) that were previously proposed on the basis of RFLP analysis of 16S rDNA (Gundersen *et al*., 1996; Jomantiene *et al*., 1998; Lee *et al*., 1998). Most of the field-collected samples were previously characterized by RFLP analysis of rDNA (Marcone *et al*., 1997b, c). Some of the periwinkle-maintained strains were previously examined by sequence- and RFLP analysis of the *tuf* gene (Schneider *et al*., 1997a).

RFLP analysis. DNA was extracted by a phytoplasma enrichment procedure as described by Ahrens & Seemüller (1992) or according to Lee *et al*. (1991, 1993a). Phytoplasmal DNA was amplified by a PCR using universal phytoplasma primers P1/P7, which amplify an approximately 1800 bp fragment extending from the 5′-end of the 16S rRNA gene to the 5′-region of the 23S rDNA (Schneider *et al*., 1995). In nested PCR assays, the P1/P7 product was employed as the template to amplify a 1240 bp fragment of the 16S rRNA gene by using universal phytoplasma primers R16F2n/R2 (Gundersen & Lee, 1996). This primer pair is a modification of pair R16F2/R2 which was previously used in phytoplasma classification, amplifying a 16S rDNA fragment of about 1245 bp in length (Lee *et al*., 1993b). The AY-specific primer pair fTufAy/rTufAy was used to amplify a 940 bp fragment of the *tuf* gene (Schneider *et al*., 1997a). RFLP analysis was performed as previously described (Lee *et al*., 1993b; Marcone *et al*., 1997a). Existing AY-group phytoplasma rDNA sequences (for references and accession numbers see Seemüller *et al*., 1998) were used to explain RFLP differences between subgroups. All nucleotide positions given below for 16S rDNA sequences correspond to positions in the sequence of strain SAY (Kuske & Kirkpatrick, 1992).

Sequence analysis. For the cloning of *tuf* gene DNA, the target DNA fragment was amplified by a PCR using universal *tuf* gene primers fTuf1/rTuf1, which yield a PCR product of approximately 1080 bp (Schneider *et al*., 1997a). Approximately 5 μg of the product was electrophoresed in a 1.5% agarose gel. Fragments with sizes corresponding to the amplified *tuf* gene sequences were excised from the gel and eluted using the QIAquick gel extraction kit (Qiagen). DNA fragments were ligated into plasmid vector pGEM-T (Promega) and recombinant plasmid used to transform *Escherichia coli* strain DH5α. Selected recombinant clones were screened for *tuf* gene inserts of the specific phytoplasmas by a PCR followed by RFLP analysis as described above. All cloned fragments showed the same RFLP profiles as the PCR-amplified DNA used for cloning.

For sequencing, plasmid DNA was purified using the NucleoSpin system kit (Macherey-Nagel). Sequencing was performed by the sequencing facility of ZMBH of the University of Heidelberg, using a dideoxy termination protocol and the ABI PRISM 310 Genetic Analyzer. The *tuf* sequences were aligned separately by using CLUSTAL, version 5, with LASERGENE software (DNASTAR). Cladistic analyses were performed with program PAUP (phylogenetic analysis using parsimony), version 3.1 (written by D. L. Swofford, University of Illinois). A phylogenetic tree was constructed by using the heuristic bisection and reconnection branch-swapping algorithm. The analyses were replicated 100 times. Sequences of the *tuf* gene of strains AAY and KVG (Schneider *et al*., 1997a) were acquired from the EMBL database (accession nos L46368 and L46369, respectively).

RESULTS AND DISCUSSION

RFLP analysis of rDNA

A total of 60 AY phytoplasma isolates maintained in periwinkle or collected from diseased plants in the field were examined (Table 1). This material was, in comparison with the 10 reference strains, differentiated and classified by RFLP analysis of the rDNA fragment amplified with primers P1/P7 followed by nested primers R16F2n/R2, using *Alu*I, *Rsa*I, *Sau*3AI, *Mse*I, *Hha*I, *Taq*I, *Hin*fI, *Hpa*II and *Hae*III restriction endonucleases. These enzymes were selected from a list of endonucleases previously used in phytoplasma classification (Lee *et al*., 1993b). However, *Sau*3AI and *Taq*I did not allow distinction of AY-group phytoplasmas when the R16F2n/R2 fragment was examined. RFLP analysis with the other enzymes revealed the presence of phytoplasmas of subgroups 16SrI-A, -B, -C, -D and -F in the material examined (Fig. 1, Table 1). Subgroups F could also be distinguished from the other subgroups by using *Rsa*I (data not shown). Subgroups E and K, represented by reference strains BBS3 and STRAWB2, were not found in the uncharacterized strains examined. In addition to the established subgroups identified, two new subgroups were recognized which were designated 16SrI-L and 16SrI-M and represented by strains AV2192 and AVUT (Fig. 1, Table 1). Both are closely related to subgroup 16SrI-B and differ from it at only one or two enzyme sites, respectively (Fig. 1; see also Table 2 for P1/P7 patterns).

When the P1/P7 fragment was used for RFLP analysis, the restriction profiles that distinguish each subgroup were consistent with those of the R16F2n/R2 fragment. However, because the P1/P7 PCR product is a longer sequence than the R16F2/R2 fragment used previously (Lee *et al*., 1993b) or the R16F2n/R2 fragment used in this work (approx. 1800 bp versus approximately 1240 bp), the diversity of the profiles between subgroups was considerably greater when the P1/P7 fragment was cleaved with the restriction enzymes described above (Figs 1, 2, 3a, 3c, 3e and 4). For example, subgroups A to F showed the same *Sau*3AI profile when the R16F2n/R2 product was examined, whereas all these groups were distinguishable using the P1/P7 fragment (Table 2, Fig. 2e). Except in the case of *Hae*III, all enzymes enabled the differentiation of at least three subgroups (Table 2). Because of the higher resolution of P1/P7 profiles, an additional subgroup, designated 16SrI-N and represented by strain IOWB, could be identified. Subgroup N is closely related to subgroup B and differs from it at only one *Hpa*II site (Table 2, Fig. 3c). Because of the greater diversity of the P1/P7 profiles, only four restriction enzymes (*Sau*3AI, *Hin*fI, *Hpa*II and *Hae*III) were needed to distinguish all nine 16SrI subgroups identified in this work. In contrast, six

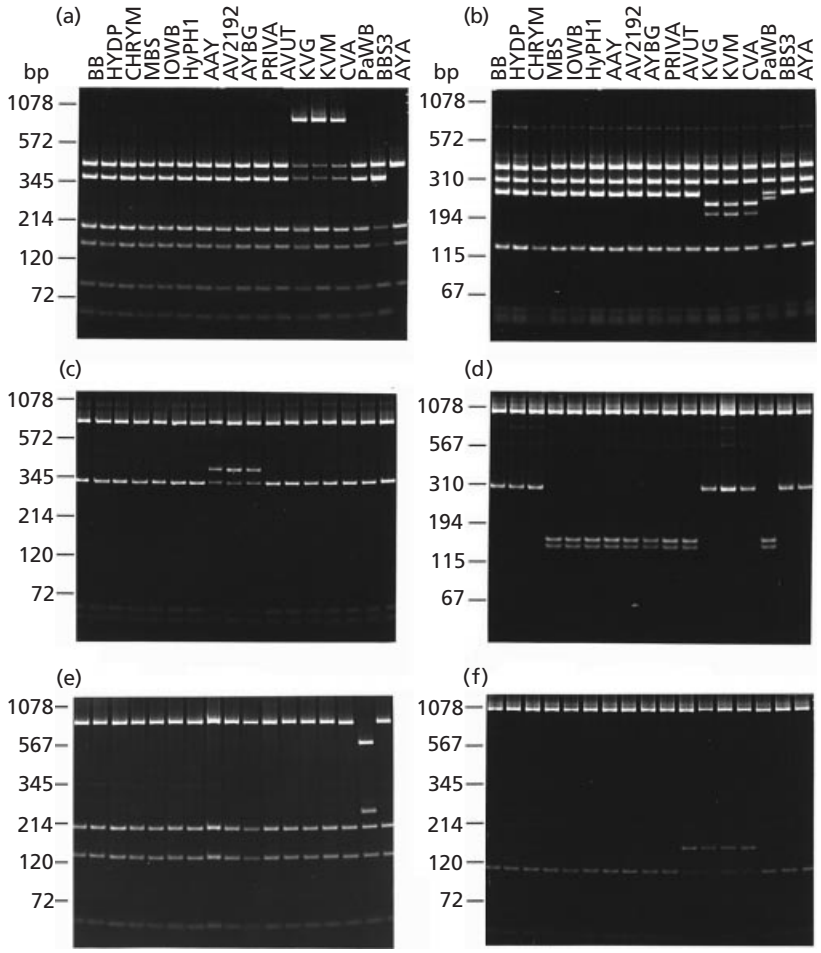

Fig. 1. *Alu*I (a), *Mse*I (b), *Hin*fI (c), *Hha*I (d), *Hpa*II (e) and *Hae*III (f) restriction profiles of rDNA from phytoplasmas of the aster yellows group that were amplified by using PCR assays with universal primer pair P1/P7 and nested by the primer pair R16F2n/R16R2. See Table 1 for phytoplasma strain abbreviations.

enzymes were necessary to distinguish seven subgroups when RFLP analysis was based on the R16F2n/R2 fragment. Finer distinctions among closely related phytoplasmas were similarly observed in RFLP analysis using the P1/P7 fragment (Jomantiene *et al*., 1998).

Many differences in the restriction profiles that distinguish the 16SrI subgroups probably can be explained by sequence heterogeneity of the two rRNA operons that seem to be present in all phytoplasmas (Schneider & Seemüller, 1994a). Such sequence heterogeneity was first suggested for clover phyllody phytoplasma on the basis of RFLP profiles (Lee *et al*., 1993b) and was described by Liefting *et al*. (1996) as occurring in the 16S rRNA genes of the phormium yellow leaf phytoplasma. On the basis of RFLP analysis, heterogeneity of the rRNA operons was also suggested to be present in an AY phytoplasma infecting *Populus alba* and *Populus tremula* (Berges *et al*., 1997) and in clover phyllody reference strain KVG of subgroup 16SrI-C (Schneider *et al*., 1997b). In our work, sequence heterogeneity was observed in subgroups 16SrI-C, -D, -E, -K, -L and -M (Table 2). Except for the unique *Mse*I profile, the P1/P7 product of the clover phyllody phytoplasma (16SrI-C) contained all of the restriction fragments that were present in subgroup A phytoplasmas. Most of the differences relative to subgroup A appear to be due to sequence heterogeneity of the two 16S rRNA genes and/or the 16S/23S rDNA spacer regions of the clover phyllody phytoplasma. For example, one 16S rRNA gene of the clover phyllody phytoplasma seems to lack the *Alu*I site at position 645, which results in the 675 bp fragment seen at the top of the profile shown in Fig. 2a. Also, the *Hae*III restriction site at position 259 appears to be missing in one 16S rRNA gene (Fig. 3e). We also suggest that one rRNA gene must possess an additional *Rsa*I site in the 424 bp fragment on top of the profile, which results in the two fragments below the third fragment from the top (Fig. 2c). In subgroup D (paulownia witches'-broom phytoplasma – PaWB), all of the differences in the rDNA profiles relative to those of subgroups A and B seem to be due to sequence heterogeneity. This is evidenced by the fact that the

Table 2. Summary of the pattern groups produced by the restriction enzymes used for RFLP analysis of 16S rDNA and 16S/23S spacer region DNA (P1/P7 fragment) from representative strains of various aster yellows phytoplasma subgroups

Numbers shown in each column represent distinct RFLP types with each restriction endonuclease.

Reference strain	16SrI subgroup	RFLP pattern type								
		*Alu*I	*Rsa*I	*Sau*3AI	*Mse*I	*Hha*I	*Taq*I	*Hin*fI	*Hpa*II	*Hae*III
BB	A	1	1	1	1	1	1	1	1	1
SAY	B	1	1	2	1*	2	2	1	2	1
KVG	C	2†	2†	3†	2	1	1	2	1	2†
PaWB	D	1	1	4†	3†	2	3†	1	3†	1
BBS3	E	3	1	5	1	3	4	3†	4	1
AYA	F	4	3	6	1	1	2	1	2	1
STRAWB2	K	5	1	7†	4	1	1‡	1	1‡	3
AV2192	L	1	1	2	1	2	2	4†	2	1
AVUT	M	1	1	2	1	2	2	4†	2	2†
IOWB	N	1	1	2	1	2	2	1	5	1

* Slight differences in the profile relative to other pattern-1 groups.

† Profile due to sequence heterogeneity of the two rRNA operons.

‡ Differences in the DNA band intensities relative to other type-1 profiles.

*Sau*3AI, *Taq*I and *Hpa*II profiles of subgroup D are composed of all fragments occurring in the subgroup A and B phytoplasmas (Figs 2e, f and 3c). However, strain PaWB does not represent a mixed infection of subgroup A and B strains. It shows, because of the heterogeneity of the two rRNA genes (Fig. 2b), a unique *Mse*I profile whereas the *Hha*I profile is composed of fragments only of subgroup B.

All of the differences between subgroup 16SrI-B and the closely related new subgroups L and M can be explained by sequence heterogeneity in the 16S rRNA genes of the new subgroups (Figs 3a and e, Table 2). Subgroups L and M, which are composed of isolates from China aster, bermudagrass and primrose, appear to lack the *Hin*fI site at position 1257 in one of the 16S rRNA genes. In addition, subgroup M, which consists of strain AVUT, seems to lack the *Hae*III site at position 259 in one 16S rRNA gene. In this respect, it is similar to the clover phyllody phytoplasma. The frequent occurrence of sequence heterogeneity between the two rRNA operons in the AY group was not known prior to our work and proved to be an important factor in 16S rDNA RFLP classification.

Both the established and the newly identified 16SrI subgroups can be distinguished by RFLP analysis of the 16S rRNA gene alone. The 16S–23S rDNA spacer region, which is contained in the P1/P7 product and which in the regions flanking the $tRNA^{Ile}$ gene has a considerably higher variability than the 16S rRNA gene (Kirkpatrick *et al.*, 1994; Smart *et al.*, 1996), did not lead to a more detailed classification. This is in contrast to the elm yellows group, in which finer distinctions were possible when the spacer region was included in RFLP analysis (Marcone *et al.*, 1997d). However, the spacer region of the AY-group phytoplasmas contributed to the diversity of the RFLP profiles and thus provided more taxonomic markers than the 16S rRNA gene alone.

RFLP analysis of the *tuf* gene

All phytoplasmas maintained in periwinkle and all field-collected samples were examined by RFLP analysis of the *tuf* gene sequence amplified with primers fTufAy/rTufAy. Digestion with *Sau*3AI, *Alu*I and *Hpa*II revealed six, five and two distinct profiles, respectively (Table 3, Figs 3b, d, f and 5). From these profiles, *tuf* gene RFLP subgroups A, B, C, D, E, F and G were defined. They show close correlation with the 16SrI subgroups: *tuf* gene RFLP subgroups A, B, C, D, E, F and G correspond to 16SrI subgroups A, B, C, E, F, K and N, respectively. In *tuf* gene RFLP subgroup A, most European isolates differed slightly, in terms of their *Alu*I profile, from the American reference strains (Table 1, Fig. 3d). Subgroups 16SrI-D, -L and -M showed the profiles of *tuf* gene RFLP subgroup B (Table 3). These subgroups are closely related to 16SrI-B and differ from it only by sequence heterogeneity of the two rRNA operons. Distinction of subgroups 16SrI-D, -L and -M from subgroup B was also not possible by digesting the fTufAy/rTufAy PCR fragment with *Rsa*I, *Mse*I, *Hha*I, *Taq*I, *Hin*fI and *Hae*III. Furthermore, *tuf* gene differentiation of other subgroups was not possible with these enzymes.

These results show that the *tuf* gene is a suitable phylogenetic and taxonomic marker. RFLP analysis of this sequence enabled the distinction of all 16SrI subgroups whose differences with respect to other subgroups are not (or not solely) based on sequence

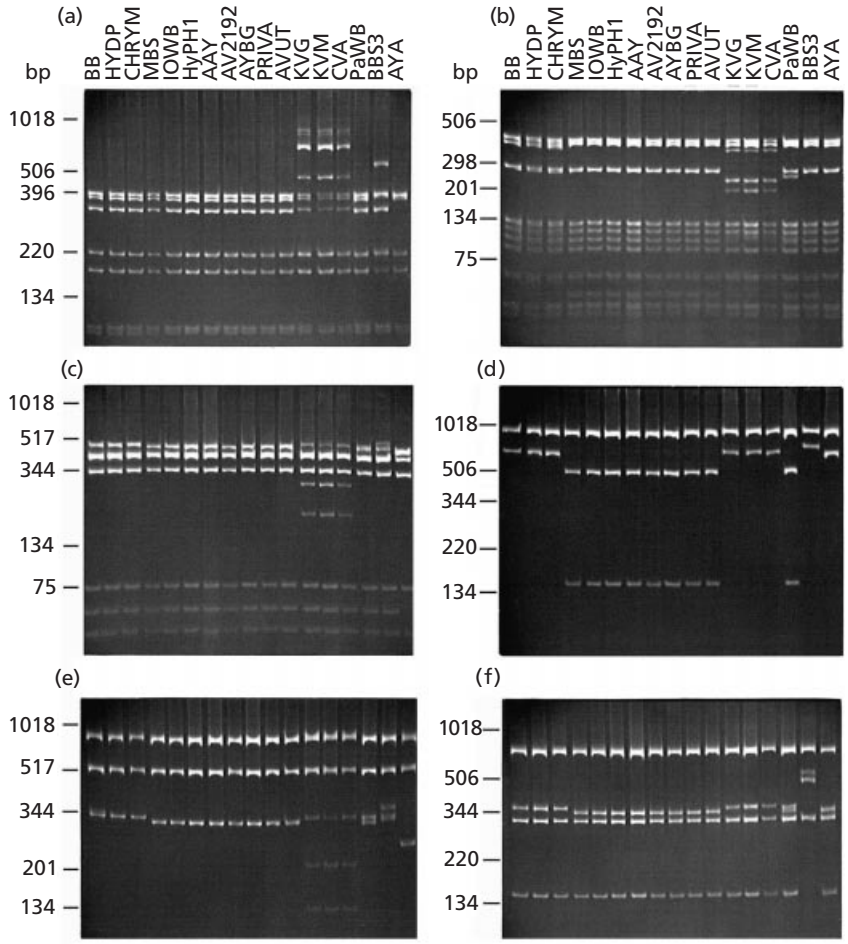

Fig. 2. *Alu*I (a), *Mse*I (b), *Rsa*I (c), *Hha*I (d), *Sau*3AI (e) and *Taq*I (f) restriction profiles of rDNA from phytoplasmas of the aster yellows group that were amplified by using PCR assays with universal primer pair P1/P7. See Table 1 for phytoplasma strain abbreviations.

heterogeneity of the two rRNA operons. In contrast, all subgroups whose differences in the rDNA RFLP profiles with respect to those of subgroups 16SrI-A and -B appear to be solely due to sequence heterogeneity of the rRNA operons could not be distinguished from subgroup 16Sr-B. This may indicate that sequence heterogeneity is a less suitable taxonomic tool than restriction sites present in both 16S rRNA genes. The results also show that the *tuf* gene, which occurs only as a single copy in mycoplasmas (Sela *et al.*, 1989; Schneider *et al.*, 1997a), has fewer RFLP markers than the often heterogeneous 16S rRNA genes of the AY phytoplasmas.

Sequence analysis of *tuf* gene

The *tuf* gene sequence in the AY group is highly conserved. The maximal difference identified by comparing 21 strains from most subgroups was 3.6% of the nucleotide positions. This is higher than that of the 16S rRNA gene, which is about 2.6% (Seemüller *et al.*, 1998; I.-M. Lee, unpublished results). The *tuf* gene sequence similarities relative to phytoplasmas of other phylogenetic groups are much lower, being approximately 88% for the stolbur phytoplasma and 75% for the apple proliferation agent, which show approximately 97 and 92.5% sequence similarity, respectively, at the 16S rDNA level (Schneider *et al.*, 1997a; Berg & Seemüller, 1999). Despite the relatively small differences within the AY group, the *tuf* gene sequence proved to be suitable for classifying these phytoplasmas. Five major clusters can clearly be distinguished, represented by reference strains BB, SAY, KVG, BBS3 and AYA (Fig. 6). These clusters and strain STRAWB2 differ from each other by at least 2.6% of the nucleotide positions. The only exception is AYA-related strain CVB, which shows greater similarity to other subgroups.

The five distinct clusters and strain STRAWB2 differentiated by sequence analysis are identical to six subgroups identified by RFLP analysis of the *tuf* gene. However, RFLP analysis of the *tuf* gene yielded an additional subgroup, represented by strain IOWB, because of a unique *Alu*I restriction profile (Fig. 3d, Table 3). On the basis of sequence analysis, strain

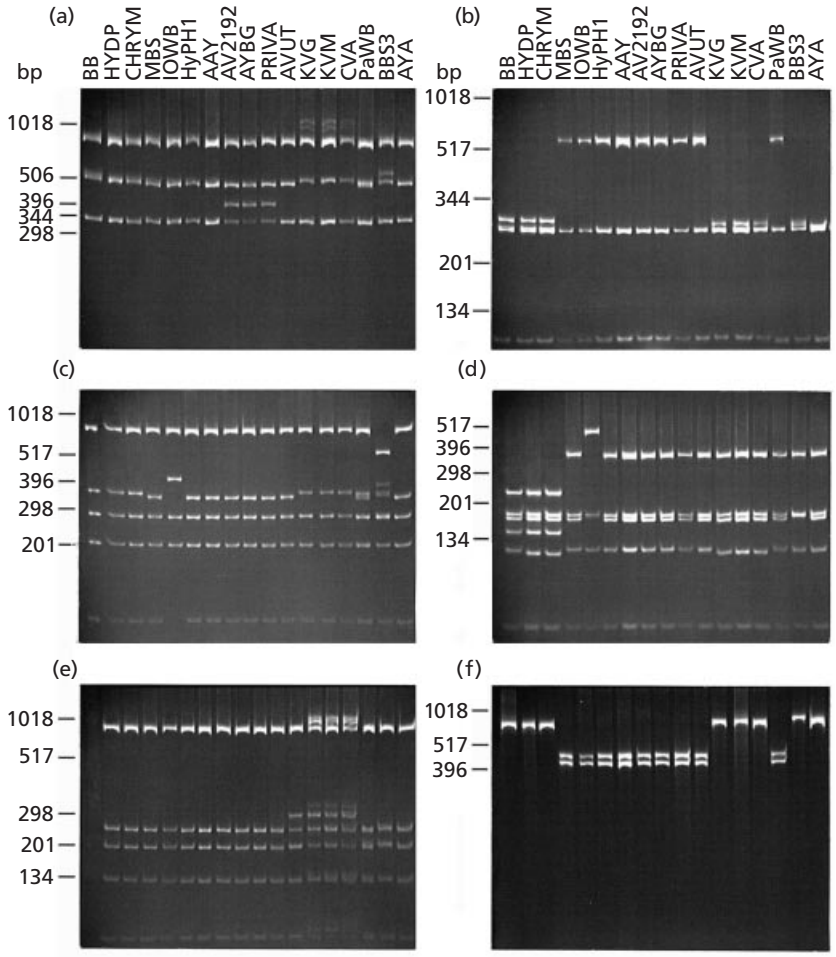

Fig. 3. *Hin*fI (a), *Hpa*II (c) and *Hae*III (e) restriction profiles of rDNA from phytoplasmas of the aster yellows group that were amplified with primer pair P1/P7. *Sau*3AI (b), *Alu*I (d) and *Hpa*II (f) restriction profiles of a phytoplasmal *tuf* gene fragment amplified with primer pair fTufAy/rTufAy. See Table 1 for phytoplasma strain abbreviations.

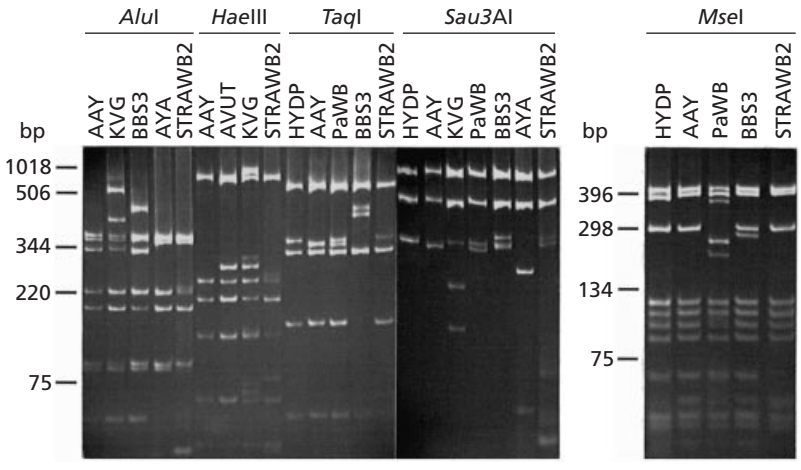

Fig. 4. *Alu*I, *Hae*III, *Taq*I, *Sau*3AI and *Mse*I restriction profiles of a rDNA fragment PCR-amplified with universal primers P1/P7 of strain STRAWB2 in comparison with strains of other AY phytoplasma subgroups. See Table 1 for phytoplasma strain abbreviations.

Table 3. Differentiation of aster yellows phytoplasmas based on RFLP analysis of PCR-amplified *tuf* gene DNA and its relationship to 16S rDNA RFLP classification

Lower-case letters shown in each line represent distinct RFLP pattern with each restriction enzyme.

Enzyme used for *tuf* gene digestion/RFLP subgroup	16SrI subgroups									
	A	B	C	D	E	F	K	L	M	N
*Sau*3AI	a	b	c	b	d	e	f	b	b	b
*Alu*I	a	b	c	b	d	b	b	b	b	e
*Hpa*II	a	b	a	b	a	a	a	b	b	b
Resulting *tuf* gene RFLP subgroup	A	B	C	B	D	E	F	B	B	G

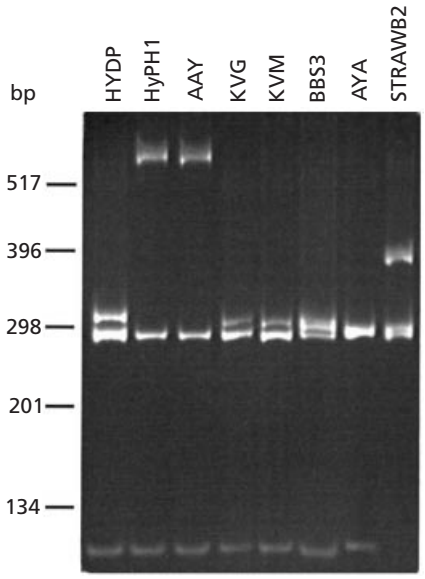

Fig. 5. *Sau*3AI restriction profile of a *tuf* gene fragment amplified with primer pair fTufAy/rTufAy of strain STRAWB2 in comparison with strains from other AY phytoplasma subgroups. See Table 1 for phytoplasma strain abbreviations.

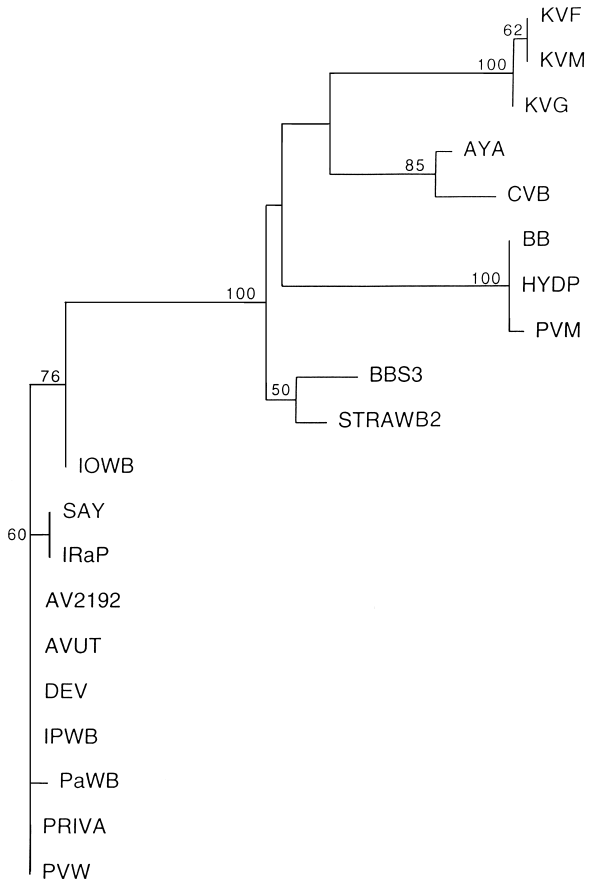

Fig. 6. Phylogenetic tree constructed by parsimony analysis of *tuf* gene sequences from 19 phytoplasmas. Branch lengths are proportional to the numbers of inferred character state transformations. Bootstrap values are shown on the branches. See Table 1 for phytoplasma strain abbreviations.

IOWB is related to the phytoplasmas of *tuf* gene RFLP subgroup B (Fig. 6). However, strain IOWB is (by branching pattern and a sequence dissimilarity of 1.0%) the most distant phytoplasma of this cluster. The maximal difference among the other strains clustering with the *tuf* gene RFLP subgroup B members is 0.6%. Strain IOWB is also distinguishable from other AY phytoplasmas by a unique *Hpa*II rDNA restriction profile (Fig. 3c, Table 2). In contrast to strain IOWB, strains PaWB, AV2192, PRIVA and AVUT showed the *tuf* gene RFLP profiles of subgroup 16SrI-B and differed only in the rDNA profiles from 16SrI-B members, resulting in subgroups 16SrI-D, -L and -M, respectively (Table 1). However, 16SrI subgroups L and M differ from 16SrI-B in only one and two restriction sites, respectively, in one rRNA operon (Figs 3a and c). In contrast, strain PaWB differs from 16SrI-B members at four restriction sites (Table 2) and by RFLP profiles of a ribosomal protein operon (Gundersen *et al*., 1996).

Conclusions

In previous work, the large and phytopathologically complex AY group could be divided into several subgroups by RFLP analysis of the rDNA, the ribosomal protein gene and *tuf* gene sequences (Gundersen *et al*., 1996; Jomantiene *et al*., 1998; Lee *et al*., 1993b, 1998; Schneider *et al*., 1997a). The various approaches yielded similar results, though the number of subgroups identified varied with the type of sequence examined. In our study, in which many strains were compared by RFLP analysis of a long rDNA fragment and by RFLP and sequence analysis of the *tuf* gene, the previous classification could largely be confirmed and consolidated. Within the AY group, six taxonomic entities can clearly be distinguished, represented by strains BB, SAY, KVG, BBS3, AYA and STRAWB2. According to the current status of phytoplasma classification, these entities represent sub-

groups of the AY phytoplasma group intended to be distinguished from other phytoplasmas at least at the species level, with the provisional prefix *Candidatus* (Bradbury, 1997). Thus, the strains listed above represent subgroups and correspond to subgroups 16SrI-A, -B, -C, -E, -F and -K, respectively, according to Lee *et al*. (1998) and Jomantiene *et al*. (1998).

Other 16SrI subgroups, as defined in this and other work (Lee *et al*., 1998), are less clearly distinguished on the basis of sequence and RFLP data from the *tuf* gene. Both rDNA and *tuf* gene data indicate that these subgroups are related to subgroup 16SrI-B. Of the subgroups in question, 16SrI-N (strain IOWB) differs most at *tuf* gene level from the typical 16SrI-B members, so its distinction from this subgroup appears to be justified. It also may be useful to distinguish subgroup 16SrI-D (the paulownia witches'-broom agent) from 16SrI-B because it differs from typical 16SrI-B members at several rDNA restriction sites (Table 2) and by a lower *tuf* gene sequence similarity. Another argument is its possible host specificity; it has never been identified in plants other than paulownia. The distinction of both 16SrI-N and 16SrI-D from other 16SrI subgroups is also supported by analysis of a ribosomal protein operon, which resulted in specific RFLP profiles for both subgroups (Gundersen *et al*., 1996). In contrast, sequence- and RFLP analysis of the *tuf* gene indicate that it is not justified to distinguish subgroups 16SrI-L and -M, which were identified in this work, from 16SrI-B. These subgroups differ little in the restriction profiles of rDNA from typical 16SrI-B members and do not differ markedly at *tuf* gene sequence level. Also, there are no pathological aspects that favour the separation of these subgroups from 16SrI-B. This result indicates that slight differences in restriction profiles should not be overestimated and that several parameters should be considered in order to obtain a useful phytoplasma classification.

The comprehensive classification of AY-group phytoplasmas attempted here and in other work (Lee *et al*., 1992, 1993b, 1998; Gundersen *et al*., 1996; Seemüller, *et al*., 1998) shows that the subgroups delineated differ considerably in geographic distribution and size. It appears that subgroup 16SrI-B is by far the most prevalent AY phytoplasma type occurring in America, Europe and Asia. Phytoplasmas of 16Sr-I subgroup A are obviously less widespread. They were thought to occur only in North America until it was established, in this and other (Lee *et al*., 1998) work, that they are also present in Europe.

The pathological relevance of the AY-group classification is not clear. The major subgroups (16SrI-A, -B and -C) have a wide host range and may also occur in the same host. Subgroups 16SrI-A and B induce in periwinkle a wide variety of symptoms such as virescence, phyllody, small and faintly coloured flowers, flower malformations, shortening of internodes, elongation and etiolation of internodes, small and deformed leaves, yellowing and decline. However, in subgroups C and E, internode elongation and etiolation are not found. To date, subgroups 16SrI-D, -E, -N and -K have been found only in one host. Although only a few strains of these subgroups have been examined, they may be host- or vector-specific.

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